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1 **Impact of muscle glycogen availability on the capacity for repeated**
2 **exercise in man**

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28 **Running head:** Carbohydrate feeding, muscle glycogen and repeated exercise

29

Abstract

Purpose: To examine whether muscle glycogen availability is associated with fatigue in a repeated exercise bout following short-term recovery.

Methods: Ten endurance-trained individuals underwent two trials in a repeated measures design, each involving an initial run to exhaustion at 70% $\dot{V}O_{2\max}$ (Run-1) followed by a 4-h recovery and a subsequent run to exhaustion at 70% $\dot{V}O_{2\max}$ (Run-2). A low-carbohydrate (L-CHO; 0.3 g·kg BM⁻¹·h⁻¹) or high-carbohydrate (H-CHO; 1.2 g·kg BM⁻¹·h⁻¹) beverage was ingested at 30-min intervals during recovery. Muscle biopsies were taken upon cessation of Run-1, post-recovery and fatigue during Run-2 in L-CHO (F2). In H-CHO, the muscle biopsies were obtained post-recovery, the time point coincident with fatigue in L-CHO (F2) and the point of fatigue during the subsequent exercise bout (F3).

Results: Run-2 was more prolonged for every participant in H-CHO (80±16 min) than L-CHO (48±11 min; $p < 0.001$). Muscle glycogen concentrations were higher at the end of recovery in H-CHO (269±84 mmol·kg dm⁻¹) *versus* L-CHO (157±37 mmol·kg dm⁻¹; $p = 0.001$). The rate of muscle glycogen degradation during Run-2 was higher in H-CHO (3.1±1.5 mmol·kg dm⁻¹·min⁻¹) than L-CHO (1.6±1.3 mmol·kg dm⁻¹·min⁻¹; $p = 0.05$). The concentration of muscle glycogen was higher in H-CHO than L-CHO at F2 (123±28 mmol·kg dm⁻¹; $p < 0.01$) but no differences were observed between treatments at the respective points of exhaustion (78±22 *versus* 72±21 mmol·kg dm⁻¹·min⁻¹; H-CHO and L-CHO, respectively).

55 **Conclusion:** Increasing carbohydrate intake during short-term recovery accelerates
56 glycogen repletion in previously exercised muscle and thus improves the capacity for
57 repeated exercise. The availability of skeletal muscle glycogen is therefore an
58 important factor in the restoration of endurance capacity because fatigue during
59 repeated exercise is associated with a critically low absolute muscle glycogen
60 concentration.

61

62 **Keywords:** Nutrition, metabolism, performance, sucrose

63

64 **Introduction**

65 Endurance capacity during an initial prolonged exercise bout is primarily reliant upon
66 pre-exercise glycogen availability, such that muscle glycogen content exhibits a direct
67 positive correlation with exercise time to exhaustion (6, 34, 36). Similarly, muscle
68 glycogen repletion can impact the time required to recover functional capacity, with
69 carbohydrate intake accelerating both glycogen resynthesis and restoration of exercise
70 capacity relative to when no carbohydrate is ingested (16, 17, 23). Furthermore,
71 carbohydrate ingestion rate exhibits a dose-dependent relationship with the rate of
72 muscle glycogen resynthesis until a threshold of ingesting $\approx 1.2 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ (8). It is
73 therefore logical to postulate that increasing carbohydrate intakes might also exhibit a
74 dose-dependent relationship with the restoration of exercise capacity following short-
75 term recovery. However, while some information is available pertaining to muscle
76 glycogen metabolism during a subsequent exercise bout (4, 9, 35), it remains merely
77 an assumption that muscle glycogen availability is an important determinant of
78 fatigue during a second bout of exercise following short-term recovery.

79

80 Based on the few studies to have examined the relationship between carbohydrate
81 ingestion rate in recovery and restoration of exercise capacity, most report no
82 consistent pattern (7, 15, 41), with only one reporting a dose-dependent relationship
83 (7). Notwithstanding that the aforementioned studies did not provide any glycogen
84 data, there is some evidence that glycogen resynthesis (in particular liver glycogen) is
85 an important determinant of endurance capacity following short-term recovery (10).
86 This is understandable given that liver glycogen content is preferentially
87 resynthesized over muscle glycogen when modest amounts of carbohydrate (≈ 0.3

g·kg BM⁻¹·h⁻¹) are ingested following an initial exhaustive exercise bout (10). Conversely, the capacity for repeated exercise has also been dissociated from skeletal muscle glycogen availability in other studies (4, 7, 9). It is therefore possible that fatigue during repeated exercise may manifest differently from an initial prolonged exercise bout, and the availability of muscle glycogen may not be the primary cause of fatigue during subsequent exercise under all conditions. Accordingly, there is an outstanding need for improved understanding about the relative importance of muscle glycogen availability in offsetting fatigue during a repeated exercise bout as opposed to an initial bout, with implications for the optimal carbohydrate feeding strategy in recovery to maximize not only glycogen resynthesis but also restoration of exercise capacity.

To this end, the current study nutritionally manipulated carbohydrate availability during short-term recovery to examine metabolic and ergogenic outcomes during subsequent exercise. Specifically, we sought to examine whether muscle glycogen availability is associated with fatigue in a repeated exercise bout following short-term recovery. Comparisons were therefore made between a low-carbohydrate (L-CHO) supplement sufficient only to restore hepatic glycogen with minimal rates of muscle glycogen resynthesis (10), and a high-carbohydrate (H-CHO) supplement designed to elicit high rates of muscle glycogen resynthesis (37). It was hypothesized that the extended run time to fatigue expected with increasing carbohydrate intake would be explained by a proportional acceleration of muscle glycogen resynthesis during recovery and thus greater glycogen availability during repeated exercise.

112 **Materials and Methods**

113 **Participants**

114 Nine healthy recreationally active men and one woman participated in the study. The
115 characteristics of this sample were: age 21 ± 1 years; body mass (BM) 72.5 ± 8.2 kg;
116 height 180 ± 9 cm; $\dot{V}O_{2\max}$ 61 ± 6 ml·kg⁻¹·min⁻¹; weekly exercise duration 5 ± 3 h.
117 The participants were informed about the possible risks and discomforts involved
118 before giving their voluntary consent to take part. The current study has been
119 approved by the local National Health Services Research Ethics Committee (Ref:
120 09/H0101/82) with a controlled clinical trial number: ISRCTN87937960.

121

122 **Preliminary measurements**

123 Participants undertook preliminary testing on two separate occasions. The first
124 preliminary visit included the determination of each participant's submaximal ($\dot{V}O_2$)
125 and maximal ($\dot{V}O_{2\max}$) oxygen uptakes (31) on a motorized treadmill (Ergo ELG70,
126 Woodway, Weil am Rhein, Germany). The data acquired from these tests were then
127 employed to calculate the treadmill speeds that elicit 60 % and 70 % of $\dot{V}O_{2\max}$. The
128 second visit (familiarization trial) was completed at least two weeks prior to the main
129 trials and required each participant to undergo the exercise protocol used in the main
130 trials (described below) without any tissue or venous blood collection, and
131 participants only ingested water at similar intervals to nutrient provision during the
132 main trials (Figure 1). This trial was aimed to accustom the participants to the
133 experimental procedures and apparatus in addition to fully familiarize with running to
134 the point of volitional exhaustion and thereby diminish any learning/trial-order effects

(Run-1 and Run-2 times to exhaustion were 103 ± 17 min and 36 ± 9 min, respectively). Expired gas samples were collected during this visit to confirm the estimated relative speeds that corresponded to the required intensity during the main trials, with any adjustments applied accordingly.

Experimental design

Each participant performed two main trials in a repeated measures experimental design interspersed by an interval of ≥ 2 weeks. A weighed dietary record was completed 48 h preceding the familiarization trial, and was subsequently repeated prior to the commencement of the main trials (2638 ± 708 kcal·d⁻¹; 55 ± 5 % carbohydrate; 17 ± 3 % fat; 28 ± 4 % protein). Participants were provided with a standardized meal (760 kcal; 57 % carbohydrate; 24 % Protein; 19 % fat) in the evening (12 ± 1 h) before the familiarization trial and replicated this prior to each main trial. Participants were also requested to abstain from alcohol consumption and refrain from strenuous physical activity for 48 h pre-trial, with any light exercise recorded and matched during the period of standardization of lifestyle for ensuing trials.

The main trials required participants to run to the point of volitional exhaustion (Run-1) at an intensity of 70 % $\dot{V}O_{2\max}$ followed by a 4 h recovery period, where participants ingested a low carbohydrate (L-CHO) or a high carbohydrate (H-CHO) supplement. Following recovery, a second run to exhaustion (Run-2) at the same exercise intensity (i.e. 70 % $\dot{V}O_{2\max}$) was undertaken by each participant to assess restoration of exercise capacity. As has been successfully applied in previous studies

that have contrasted relative and absolute fatigue points to understand fatigue mechanisms in relation to running (34) and cycling (12), trial order required L-CHO to be completed first. Previous data (7) has reported that the restoration of exercise capacity can be dose-dependent with ingestion of moderate-high *versus* high carbohydrate during short-term recovery. Differences in exercise time to exhaustion can therefore be confidently expected between the more markedly different very low *versus* high carbohydrate doses in this study. Accordingly, establishing the absolute time-point of fatigue in L-CHO trials prior to H-CHO trials enables comparisons in the metabolic environment both at the point of volitional fatigue in both treatments and at the time point in the H-CHO treatment that corresponds to fatigue during L-CHO treatment.

Consistent with the above rationale, muscle biopsy samples were obtained in L-CHO trial: upon cessation of Run-1; post-recovery; and volitional exhaustion during Run-2 (F2). In the H-CHO trial, the three muscle biopsy samples were obtained: post-recovery; the time point coincident with fatigue in L-CHO (F2); and the point of volitional exhaustion during the subsequent exercise bout (F3). As a result of the dietary and activity standardization, and the fact the participants ran to the point of volitional exhaustion, negligible intra-individual variability in muscle glycogen levels following Run-1 were expected between trials, as previously reported in a similar protocol (34) and this was further verified by the well-matched times to exhaustion during Run-1 in both trials (results section). Thus, the sample obtained following Run-1 in L-CHO merely serves to verify the expected substantial glycogen depletion from the exercise protocol, whilst the remaining samples across both trials inform the

primary research questions pertaining to differences in muscle glycogen availability immediately prior to and during the second exercise bout.

Experimental protocol

The experimental protocol pertaining to the current study is described in further detail elsewhere (1). Each participant arrived to the laboratory at 08:00 \pm 1 h following an overnight fast (\geq 10 h). Upon arrival at the laboratory, participants completed a profile of mood state (POMS) questionnaire, before a baseline urine sample was obtained. Post-void nude body mass (BM) was then recorded (Weylux 424, UK) before a 5 min resting expired gas sample was collected using the Douglas bag technique. An indwelling cannula was inserted into an antecubital vein and a 10 ml baseline venous blood sample was collected. Participants commenced the exercise protocol with a standardized 5 min warm-up at 60 % $\dot{V}O_{2\max}$, where speed was then increased to 70% $\dot{V}O_{2\max}$ until the point of volitional exhaustion (11 ± 1 km·h⁻¹). During Run-1, one minute expired gas samples, heart rate (HR; Polar FT2, Kempele, Finland), ratings of perceived exertion (RPE), and 10 ml blood samples were collected (Figure 1). Water intake was permitted *ad libitum* during the familiarization trial (0.5 ± 0.3 L during Run-1) and then matched for subsequent trials. To accurately gauge relative levels of fatigue, participants were permitted to reduce the intensity (walking at 4.4 km·h⁻¹) for 2 min intervals on two occasions when they indicated that they could not maintain the prescribed intensity, followed by a return to the treadmill speed equivalent to 70 % $\dot{V}O_{2\max}$. Only on the third occasion that participants indicated that they were unable to run at the prescribed speed was volitional exhaustion accepted. Immediately following Run-1 in L-CHO trial, participants rested on an adjacent bed in a semi-

207 supine position while ≈ 80 mg of muscle was obtained from the *vastus lateralis* by
208 percutaneous needle biopsy technique (5) from a 3-5 mm incision made prior to
209 exercise at the anterior aspect of the thigh using a surgical blade under local anesthetic
210 (1 % lidocaine; Hameln Pharmaceuticals Ltd., Brockworth, UK). Thereafter, the first
211 bolus of prescribed solution was immediately provided and recovery time
212 commenced, before nude BM mass was recorded to assess hydration status through
213 percentage change in mass (with body mass adjusted for the ingested bolus). The
214 remaining seven aliquots of prescribed solution were provided at 30 min intervals
215 (Figure 1). Participants were permitted 15 min to consume each volume, and
216 subjective measures of stomach discomfort, gut fullness and thirst were recorded
217 following the ingestion of each bolus using adapted Borg scales (1). Expired gas
218 followed by venous blood samples were collected hourly prior to fluid provision.
219 Furthermore, urine output was collected throughout the 4 h recovery period.
220 Approximately 3 h 40 min into recovery, two (in L-CHO trial) or three (in H-CHO
221 trial) 3-5 mm incisions were made proximally on the same leg at least 3 cm apart
222 followed by obtaining a muscle biopsy sample at the end of recovery (with the
223 remaining incisions dressed for easy access at later sampling points), with the order of
224 dominant/non-dominant legs for muscle biopsy sampling being counterbalanced
225 between the main trials. Nude BM was recorded at the end of recovery, and
226 participants initiated a standardized warm-up before running at 70 % $\dot{V}O_{2\max}$ to
227 volitional exhaustion. As for Run-1, water intake was permitted *ad libitum* during the
228 familiarization trial and matched for subsequent trials (0.3 ± 0.3 L during Run-2).
229 Reaching the point of volitional exhaustion was determined in an identical manner to
230 the initial exercise bout. Expired gases, HR, RPE and venous blood samples were also
231 collected during Run-2 (Figure 1). In the L-CHO trial fatigue was reached after $48 \pm$

11 min, at which point the one remaining incision site in that trial was used to obtain a final muscle biopsy. Therefore, after 48 ± 11 min in the subsequent H-CHO trial, the exercise protocol was briefly (624 ± 236 seconds) interrupted to obtain a muscle biopsy at the time point coincident with fatigue during L-CHO trial (i.e. F2) – thus permitting comparison of glycogen concentrations at a matched absolute time-point and rate of degradation over a matched period between the two nutritional interventions, as employed previously (34). Participants then mounted the treadmill and continued to run until volitional exhaustion before the final biopsy (i.e. F3) was obtained from the remaining incision site. BM was subsequently recorded following the attainment of the final biopsy from each participant. Ambient temperature and humidity were recorded at 60 min intervals throughout the trials using a portable weather station (WS 6730; Technoline, Berlin, Germany) and were not different among the trials: 20.3 ± 0.5 and $20.1 \pm 0.5^\circ\text{C}$; and 46 ± 2 and 47 ± 2 % in L-CHO and H-CHO trials, respectively. Background music was standardized between trials and participants were unaware of the time elapsed during the exercise capacity test, with all verbal encouragement standardized.

Solution composition

The rates of carbohydrate (sucrose) intake in the L-CHO and H-CHO trials were $0.3 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$ and $1.2 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$, equating to a total amount of carbohydrate provided during the recovery period of $87 \pm 10 \text{ g}$ and $349 \pm 41 \text{ g}$ in L-CHO and H-CHO beverages, respectively. All treatment solutions were isovolumetric ($10 \text{ ml}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$) relative to each participant's BM ($727 \pm 86 \text{ ml}\cdot\text{h}^{-1}$), thus formulating a 3 % and 12 % solution in L-CHO and H-CHO respectively. Both supplements were

matched for their electrolyte content (sodium and potassium) and were flavor matched. Full information pertaining to the nutritional treatments is provided elsewhere (1). Owing to the design of the experiment (i.e. participants were aware of the number of biopsies planned during each run), the treatments were not blinded.

Blood analysis

From each 10 ml venous blood sample, 5 ml was transferred into a non-anticoagulant tube and left to clot for \approx 45-min at room temperature before being centrifuged at 2000 xg for 10 min at 4°C (Heraeus Primo R; Thermo Fisher Scientific, Loughborough, UK) for the analysis of serum insulin concentrations via enzyme-linked immunosorbent assay (ELISA; Mercodia, Uppsala, Sweden) using a spectrophotometric plate reader (Spectrostar Nano, BMG Labtech, Ortenberg, Germany). The remaining 5 ml of each blood sample was dispensed into a ethylenediaminetetraacetic acid (EDTA) treated tube and was immediately analyzed for hemoglobin (Sysmex SF-3000 Sysmex Ltd., Wymbush, UK) and hematocrit (Hawksley, Lancing, UK) concentrations for the determination of plasma volume changes throughout the trials (14). The remaining blood was then spun for centrifugation under 2000 xg for 10 min at 4°C for the analysis of plasma glucose, non-esterified fatty acids, lactate and urea using a spectrophotometric analyzer (RX Daytona, Randox Laboratories Ltd., Crumlin, UK).

Muscle analysis

Each muscle sample was immediately extracted from the needle biopsy and snap-frozen into liquid nitrogen, where it was subsequently dissected to remove 15-30 mg

of muscle fragment prior to being placed in a freeze-dryer (Modulyo, Edwards, UK) for ≈ 18 hours at -50°C . After removal of visible blood and connective tissue, the freeze-dried muscle samples were then reduced to fine powder using an agate pestle and mortar and used for the extraction and determination of phosphocreatine (PCr), creatine (Cr) and muscle glycogen concentrations. The relative concentrations of these metabolites were determined in duplicate according to enzymatic methods previously described (18, 26, 34) using a spectrophotometric plate reader (SpectraMax 190, Molecular Devices, USA). Glycogen was assayed by hydrolysis in 1 mol/l hydrochloric acid (HCl) and was determined both as acid-soluble and acid-insoluble glycogen (22). The total mixed-muscle glycogen concentration was calculated by adding the acid-soluble and acid-insoluble glycogen concentrations. All muscle metabolites were adjusted to peak total Cr (PCr+Cr) for each subject to correct for variability in blood, connective tissue, and other non-muscle constituents between biopsies. Total glycogen concentrations are reported as mmol glucosyl units per kilogram of dry mass ($\text{mmol}\cdot\text{kg}^{-1}\cdot\text{dm}^{-1}$) to account for any measurement error associated with fluid shift during exercise and rehydration. The contribution of muscle glycogen towards whole-body carbohydrate oxidation during Run-2 was estimated from lean tissue mass of all leg muscle (6 % of body mass) from a typical 72.1 kg trained individual using dual-energy X-ray absorptiometry (DXA) analysis (9).

Expired gas analysis

Expired gas samples were collected using the Douglas bag method (Hans Rudolph, Shawnee, KS, USA), and the relative oxygen and carbon dioxide fractions were quantified by paramagnetic and infrared analyzers, respectively (Servomex,

Crowborough, UK). The calculations of $\dot{V}O_2$ and $\dot{V}CO_2$ were then used for the determination of carbohydrate and lipid oxidation rates ($g \cdot min^{-1}$) using stoichiometric formulae, assuming that the contribution of protein oxidation was negligible under those conditions (24):

$$\text{Carbohydrate Oxidation} = (4.210 \cdot \dot{V}CO_2) - (2.962 \cdot \dot{V}O_2)$$

$$\text{Fatty Acid Oxidation} = (1.695 \cdot \dot{V}O_2) - (1.701 \cdot \dot{V}CO_2)$$

Extra-muscular carbohydrate oxidation was then derived from the difference between whole-body carbohydrate oxidation as determined from indirect calorimetry and intramuscular carbohydrate oxidation (overall muscle glycogen degradation rates).

Urine analysis

Baseline urine collection to determine hydration was assessed via freezing point depression method by using a cryoscopic osmometer (Advanced Instruments, Inc, Norwood, MA, USA) and adequate hydration was assumed for osmolality values $\leq 900 \text{ mOsm} \cdot \text{kg}^{-1}$ (30). During the 4 h recovery period, the voided urine was collected in a vessel for the determination of total urine output during recovery.

POMS questionnaire

On the day of each trial, before exercise, participants completed a 37-item short form profile of mood state (POMS-SF) questionnaire (28). POMS-SF items are divided into six categories: tension, depression, anger, fatigue, confusion and vigor. Total mood disturbance (TDM) was then calculated as the sum of the first five categories minus vigor.

327

328 **Statistical analysis**

329 *A priori* sample size estimation was conducted based on the exercise capacity data of
330 a similar previous study (7) which showed that a sample size of $n=6$ would provide
331 90% power to detect a difference in exercise capacity of 16.2 min using a two-tailed *t*-
332 test between two carbohydrate supplements with differing amounts. Paired *t*-tests
333 were used to analyze data involving a single comparison of two level means. Where
334 paired-difference data were deemed non-normally distributed by Shapiro-Wilk test,
335 values are reported as median (range), with Wilcoxon signed rank test being used to
336 compare medians. A two-way linear mixed model with repeated measures (time x
337 trial) was employed to identify overall differences between experimental conditions.
338 Wherever a significant interaction effect was found, a Bonferroni step-wise correction
339 was employed to determine the location of the variance (3). Pearson product moment
340 correlation coefficient (*r*) was used to explore the relationship between muscle
341 glycogen availability at the end of recovery and time to exhaustion during Run-2.
342 Incremental area under the concentration curve (iAUC) for plasma glucose and serum
343 insulin concentrations during the recovery were calculated using the method
344 recommended by Wolever (40). Statistical procedures were performed using
345 commercially available software (IBM SPSS version 21.0, SPSS Inc., Chicago, IL)
346 and significance was set at an alpha level of 0.05. Unless otherwise stated, all results
347 were reported in text as the mean \pm standard deviation (SD) of the mean and the error
348 bars depicted in figures are confidence intervals (CI) that have been corrected to
349 remove between-subject variance (25).

350

351 **Results**

352 **Exercise capacity**

353 The run times to exhaustion in Run-1 (i.e. prior to intervention) were very well-
354 matched between treatments, with median time to exhaustion of 105 min (72-133
355 min) in L-CHO trial and 105 min (75-161 min) in H-CHO trial ($p= 0.12$). All
356 participants were able to run longer during the subsequent run when more
357 carbohydrate had been ingested in recovery, with mean run times of 48 ± 11 min in L-
358 CHO and 80 ± 16 min in H-CHO ($p < 0.001$). Moreover, the magnitude of this pattern
359 between treatments was consistent for every participant in the study (i.e. improvement
360 of 31 ± 9 min), as represented in Figure 2.

361

362 Relative exercise intensities were also successfully standardized between the
363 experimental treatments and averaged 69 ± 1 % $\dot{V}O_{2\max}$ in Run-1 and 69 ± 3 %
364 $\dot{V}O_{2\max}$ in Run-2 across both treatments. These were reflected by the overall heart
365 rates of 169 ± 9 and 167 ± 9 beats \cdot min $^{-1}$ recorded during L-CHO and H-CHO,
366 respectively.

367

368 **Muscle glycogen**

369 Figure 2 illustrates muscle glycogen concentrations across both treatments. A time x
370 trial interaction was established for total muscle glycogen concentrations ($F= 9.8$; $p=$
371 0.003) and accordingly there was greater muscle glycogen content at the end of
372 recovery in H-CHO than L-CHO. Despite a higher rate of glycogen degradation
373 during Run-2 in the H-CHO treatment (3.1 ± 1.5 mmol \cdot kg $^{-1}\cdot$ min $^{-1}$), when

compared to the absolute fatigue time point in L-CHO trial ($1.6 \pm 1.3 \text{ mmol} \cdot \text{kg dm}^{-1} \cdot \text{min}^{-1}$ ($p = 0.05$) the muscle glycogen concentration at F2 was still higher in the former trial ($123 \pm 28 \text{ mmol} \cdot \text{kg dm}^{-1}$ *versus* $72 \pm 21 \text{ mmol} \cdot \text{kg dm}^{-1}$; $p < 0.01$). Muscle glycogen concentrations were reduced to similar levels at the point of volitional exhaustion in both trials (Figure 2). A significant correlation was established ($r = 0.45$; $p = 0.045$) between muscle glycogen content at the end of recovery and time to exhaustion during Run-2.

Plasma glucose and NEFA

A time x trial interaction was observed in plasma glucose during recovery ($F = 8.65$; $p = 0.004$; Figure 3), which was associated with a higher glycemic iAUC in H-CHO ($299 \pm 125 \text{ mmol} \cdot 240 \text{ min} \cdot \text{l}^{-1}$) during recovery than L-CHO ($180 \pm 138 \text{ mmol} \cdot 240 \text{ min} \cdot \text{l}^{-1}$; $p = 0.04$). There were also notable differences during the subsequent run ($F = 5.63$; $p = 0.02$), with slightly lower plasma glucose concentrations in H-CHO than L-CHO in the initial 30 min of exercise. No frank hypoglycemia was observed at the point of fatigue in L-CHO ($4.9 \pm 1.1 \text{ mmol} \cdot \text{l}^{-1}$) or H-CHO ($5.0 \pm 0.9 \text{ mmol} \cdot \text{l}^{-1}$).

Plasma NEFA concentrations were rapidly suppressed to basal levels during recovery in H-CHO while maintained at a relatively higher level in the L-CHO trial (treatment: $p = 0.04$). Upon commencement of the subsequent run, plasma NEFA were consistently elevated in L-CHO when compared to H-CHO (treatment: $p < 0.001$). An increase in NEFA concentrations from F2 to F3 was observed in H-CHO trial ($p = 0.008$, Figure 4).

Serum insulin

Serum insulin concentrations were significantly higher during recovery when H-CHO was ingested as opposed to L-CHO ($F= 9.0$; $p= 0.004$; Figure 2). Accordingly, the insulinemic iAUC for the entire 4-h recovery period was elevated threefold when H-CHO was ingested when compared to L-CHO (28 ± 12 *versus* 7 ± 3 $\text{nmol} \cdot 240 \text{ min} \cdot \text{l}^{-1}$; $p= 0.02$).

Plasma lactate and urea

Plasma lactate concentrations declined during recovery in L-CHO but remained relatively elevated in H-CHO (time: $p= 0.005$). However, plasma lactate levels during the subsequent run (Figure 4) were not dissimilar between in L-CHO and H-CHO (2.5 ± 0.3 and 2.6 ± 0.2 $\text{mmol} \cdot \text{l}^{-1}$, respectively; $p= 0.6$). The plasma concentration of urea was not different between treatments and remained at basal levels throughout trials (5.6 ± 0.4 $\text{mmol} \cdot \text{l}^{-1}$ in both treatments).

Substrate metabolism

Whole-body carbohydrate and lipid oxidation rates were substantially different between treatments during Run-2 ($F= 7.96$; $p= 0.006$; Table 1). Although overall rates of metabolism during the repeated exercise bout were similar between treatments (L-CHO = 64.9 $\text{kJ} \cdot \text{min}^{-1}$; H-CHO = 66.7 $\text{kJ} \cdot \text{min}^{-1}$), H-CHO ingestion resulted in lower lipid oxidation rates than L-CHO (4.3 ± 2.2 vs. 11.2 ± 3.5 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; $p< 0.001$) but higher rates of carbohydrate oxidation (44.5 ± 6.5 vs. 25.2 ± 9.3 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, respectively; $p<0.001$). Figure 5 illustrates that the higher rates of whole body carbohydrate oxidation in H-CHO trial were likely attributable to variations in

glycogen rather than extra-muscular carbohydrate metabolism (e.g. glucose and lactate), both at the point corresponding to fatigue with L-CHO (F2) and the point of absolute fatigue (F3).

Hydration and subjective data

Pre-exercise hydration status verified adequate fluid balance and was not different between treatments, as indicated by urine osmolality of 496 ± 316 and 540 ± 266 mOsm.kg⁻¹ in L-CHO and H-CHO, respectively ($p= 0.5$). Changes in BM were similar ($p= 0.6$) across both trials (-1.2 ± 0.6 and -1.3 ± 0.6 kg in L-CHO and H-CHO, respectively). The change in plasma volume was also similar ($p= 0.9$) between the respective treatments (2 ± 3 vs. 1.8 ± 3 %, respectively). The total urine produced during recovery was 1749 ± 840 ml in L-CHO and 1247 ± 613 ml in H-CHO trials ($p= 0.09$). There were no differences in any of the mood state categories in the POMS-SF ($p> 0.05$). A significant time \times trial interaction was observed in RPE ($F= 6.38$; $p= 0.01$); participants' perceived effort was significantly lower in H-CHO than L-CHO from 15 min until F2 during Run-2 ($p< 0.05$). Subjective ratings of gut fullness, thirst, and stomach discomfort were similar between the experimental conditions (data not shown).

Discussion

The experimental design presented here provides novel insight regarding the role of muscle glycogen in fatigue by enabling both time- and fatigue-matched comparisons of substrate availability and utilization during the late stages of repeated exercise. Effective standardization of other relevant variables lends direct support to the

hypothesis that muscle glycogen availability after recovery from prior exercise is indeed a primary determinant of subsequent exercise capacity. From a practical perspective, having utilized nutritional manipulation of carbohydrate availability to understand the role of glycogen, it can also therefore be concluded that carbohydrate ingestion can be employed to impact repeated exercise capacity via this mechanism.

The improvement in subsequent endurance capacity with H-CHO treatment was clearly demonstrated by an increase of 31 ± 9 min relative to L-CHO, which is in agreement with one previous experiment (7) but in contrast with two others (15, 41). These discrepancies may be a consequence of a number of factors. The current study in addition to that of Betts et al. (7) included younger participants with higher $\dot{V}O_{2\max}$ than those used in previous investigations (15, 41). Furthermore, we employed a familiarization trial that was identical to the main experimental procedures. These measures may be an important distinction when considering that aerobically trained individuals who are familiarized with exercise capacity testing may be necessary to detect small, worthwhile intervention effects (19). Moreover, subtle differences in the current experimental procedures may have contributed to accurately reaching true volitional exhaustion. Specifically, participants in the current experiment, as well as the only other study reporting a dose-dependent improvement in exercise capacity with carbohydrate ingestion (7), reduced the intensity on two occasions before fatigue was accepted. Indeed, participants were able to run for 10 ± 4 min from the first walk until the point of exhaustion in this study, enforcing the notion that volitional exhaustion may not have been achieved in previous investigations that did not allow these walks. Of course, other differences between protocols such as the precise type,

amount, and/or feeding frequency of the ingested carbohydrate offer possible alternative explanations (15, 41).

Ingestion of $1.2 \text{ g sucrose} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ markedly increased muscle glycogen availability compared to the relatively low quantity of sucrose ($0.3 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). This finding is consistent with most previous studies investigating muscle glycogen restoration with differing amounts of carbohydrate (8). In the current experiment, muscle glycogen utilization was accelerated with higher carbohydrate intake and thus glycogenolysis was shown to be proportional to muscle glycogen concentration, as has previously been determined (2, 29). Nevertheless, similar rates of muscle glycogen utilization were reported during a repeated exercise bout when differing amounts of carbohydrate were ingested during recovery (4, 35). The precise reasons for these apparently discrepant findings in relation to muscle glycogen utilization may be ascribed to the use of ^{13}C -magnetic resonance spectroscopy by Berardi et al. (2006) to quantify muscle glycogen degradation (i.e. wider musculature *versus* biochemical analysis of $<100 \text{ mg}$ from the *vastus lateralis*; although these techniques correlate well (32)) and the type of exercise performed (i.e. cycling) that were dissimilar from the present study. Equally, the study by Tsintzas et al. (2003) employed treadmill running during a non-exhaustive exercise bout (15 min) and provided lower amounts of carbohydrate to the current experiment ($0.15 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ *versus* $0.53 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). Concurrent with our finding that muscle glycogen concentrations were reduced to similar levels at the point of volitional exhaustion across both treatments, the current data suggest that muscle glycogen availability *per se* was associated with the improved restoration of endurance capacity with increased carbohydrate intake.

When interpreting the ergogenic effect with H-CHO ingestion, it is important to consider the brief period where exercise was interrupted in this trial to obtain a muscle biopsy sample to compare glycogen utilization at this fatigue-matching point relative to L-CHO. Muscle glycogen restoration would occur at very low rates in the absence of carbohydrate feeding ($\approx 0.5 \text{ mmol} \cdot \text{kg dry mass}^{-1} \cdot \text{min}^{-1}$ (21)). During a subsequent exercise bout at similar intensities, muscle glycogen utilization was estimated to be $\approx 2.5 \text{ mmol} \cdot \text{kg dry mass}^{-1} \cdot \text{min}^{-1}$ during treadmill running (35). Thus, any resynthesis that may have occurred during the brief interruption period (624 ± 236 seconds) would theoretically account for only 2 min of extended exercise. Other possibilities that may have influenced subsequent exercise capacity in H-CHO treatment include knowledge of the treatment order and the psychological impact of resting period to obtain the muscle biopsy. Nevertheless, regarding the former, it was previously demonstrated that there was no placebo effect when carbohydrate was ingested during prolonged cycling, and that there was a clear ergogenic effect with carbohydrate intake relative to both a placebo and water ingestion (20). In relation to the psychological effect of the brief period to obtain the muscle sample, it was apparent that participants were able to continue exercising during H-CHO (RPE; 16 ± 1) relative to the fatigue-matching point (i.e. F2) in L-CHO (20 ± 0) and thus indicating that participants' perceived effort was lower in H-CHO than L-CHO treatment before exercise was interrupted in H-CHO to obtain the final biopsy sample. When considered collectively, it is reasonable to affirm that the short period to obtain a muscle sample is unlikely to explain the 65 % improvement in the capacity for subsequent exercise and that the imposed nutritional intervention may be ascribed for the ergogenic effect with H-CHO intake.

The lowering of blood glucose was more prominent in H-CHO during the initial 30 min of the subsequent run, likely reflecting a transient increase in leg glucose uptake and reduced liver glucose output secondary to the increase in insulin concentrations (27). Conversely, the relatively elevated plasma glucose concentrations early in exercise in the L-CHO trial likely reflect an increased hepatic glucose output, which is predominantly supported by an increased rate of hepatic glycogenolysis (38). Thus, the increased insulinemimetic response during recovery in H-CHO may have initially spared liver glycogenolysis such that glucose production to the active muscles was possible late in exercise. These physiological responses coupled with our finding of limited muscle glycogen restoration in L-CHO supports our prior assumption that the modest amounts of ingested carbohydrate will be largely sequestered by the liver due to highly efficient first pass hepatic extraction (10, 39). It is likely that liver glycogen resynthesis was augmented in both trials owing to the presence of fructose in the sucrose solutions (13) and thus the ongoing absorption of the ingested carbohydrate in H-CHO treatment is likely to contribute to the observed higher carbohydrate oxidation with this treatment. Indeed, both liver and muscle glycogen have an important role in restoration of subsequent endurance capacity (10). Therefore, it is not unreasonable to suggest that liver glycogen availability and increased exogenous carbohydrate oxidation may have contributed to the overall effect in the H-CHO treatment. Nonetheless, estimations of extra-muscular carbohydrate oxidation were not different between F2 and F3 in the H-CHO treatment (Figure 5). In conjunction with the observation of an increased glycogen utilization rate with H-CHO and that fatigue in both treatments coincided with depletion of muscle glycogen to critically low muscle glycogen concentrations, the current findings demonstrate that the

availability of muscle glycogen is a primary determinant of fatigue during a repeated exercise bout following short-term recovery.

Hypoglycemia and subsequent reduction in carbohydrate oxidation late in exercise have been proposed as a major cause of fatigue during prolonged moderate to high-intensity cycling exercise (12). However, it has been consistently demonstrated that fatigue during prolonged moderate to high-intensity running is not associated with hypoglycemia (33, 34, 36). The latter notion was further supported by the current investigation, whereby fatigue was not associated with hypoglycemia in either treatment. Additionally, whilst carbohydrate oxidation during a repeated exercise bout was greater when higher amounts of carbohydrate ($\approx 0.75 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) were provided relative to a lower dose ($\approx 0.25 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) during recovery, no discernable differences in plasma glucose concentrations or time to exhaustion were shown (15). Indeed, fatigue during prolonged exercise was shown independent of carbohydrate oxidation or avoidance of hypoglycemia (11). Further support for the latter study comes from H-CHO trial in the present investigation, where neither hypoglycemia nor a decline in carbohydrate oxidation was apparent at the cessation of exercise to explain fatigue. Thus, it can be suggested that factors other than hypoglycemia or a decline in carbohydrate oxidation rates limited the capacity for subsequent exercise.

In conclusion, the ingestion of $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of carbohydrate during 4 h recovery from an initial exhaustive exercise bout increased muscle glycogen availability prior to a repeated exercise bout when compared with the ingestion of $0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. In concordance, the capacity for repeated exercise was improved in a dose-dependent

manner. The rate of glycogen utilization was accelerated in the H-CHO trial during the repeated exercise bout and fatigue was associated with glycogen depletion to critically low levels in both treatments. The extended run time to fatigue expected with increasing carbohydrate intake is attributable to increased muscle glycogen repletion during recovery and therefore the availability of this substrate during Run-2.

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691 **Table 1. Substrate metabolism and respiratory exchange ratio (RER) during Run-1 and Run-2 with L-CHO or H-CHO treatments.**

	Run-1					Run-2					
	Pre	30 min	60 min	90 min	F1	15 min	30 min	45 min	F2	60 min	F3
Carbohydrate oxidation (g·min⁻¹)											
L-CHO	0.26±0.15	2.20±0.36	2.18±0.37	1.90 ±0.40	1.87±0.72	1.92±0.74	1.99±0.89	2.74±1.04	1.60±0.79		
						*	*		*		#
H-CHO	0.33±0.19	2.59±0.70	2.30±0.59	2.02±0.80	1.98±0.73	2.68±0.68	3.18±1.06	2.81±0.95	2.41±0.46	2.66±0.47	2.41±0.98
Lipid oxidation (g·min⁻¹)											
L-CHO	0.06±0.06	0.57±0.20	0.61±0.21	0.76±0.22	0.77±0.36	0.77±0.22	0.71±0.31	0.56±0.31	0.94±0.24		
						*	*		*		#
H-CHO	0.06±0.07	0.44±0.20	0.57±0.23	0.66±0.44	0.70±0.32	0.24±0.17	0.26±0.18	0.40±0.26	0.45±0.16	0.36±0.23	0.50±0.33
RER											
L-CHO	0.87±0.12	0.89± 0.04	0.88±0.04	0.85±0.04	0.85±0.06	0.85±0.05	0.86±0.07	0.90±0.07	0.82±0.05		
						*	*		*		#
H-CHO	0.90±0.11	0.91±0.04	0.89±0.04	0.88±0.08	0.86±0.06	0.96±0.05	0.95±0.04	0.92±0.05	0.91±0.04	0.93±0.02	0.90±0.06

692 **Values are mean ± SD. *, values different between L-CHO and H-CHO (p< 0.05); #, values different at absolute fatigue (F2 vs. F3)**

693 **between L-CHO and H-CHO (p<0.05)**

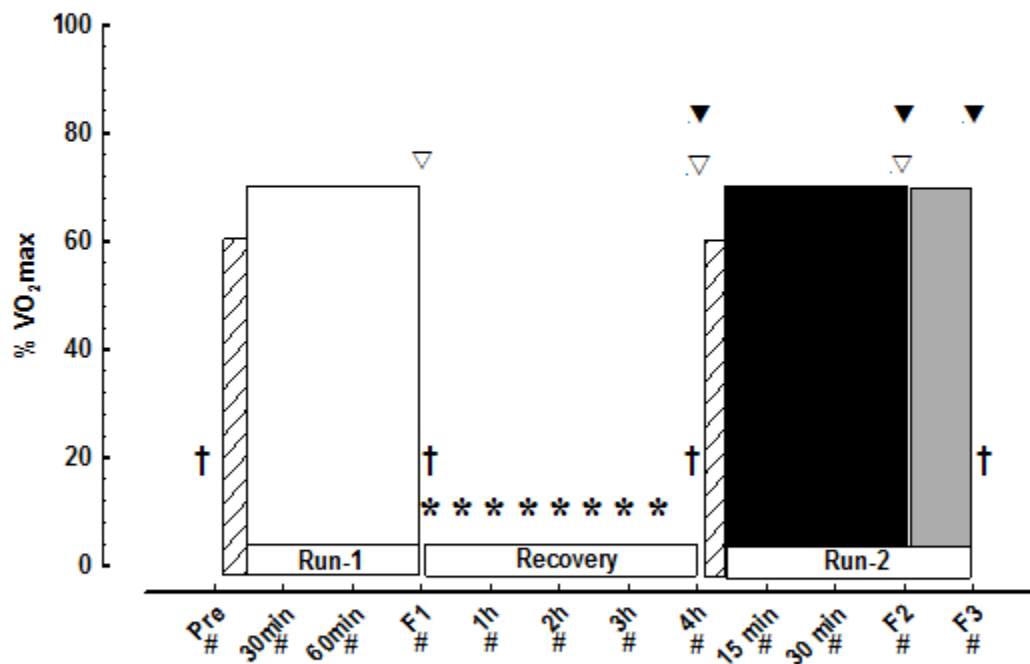
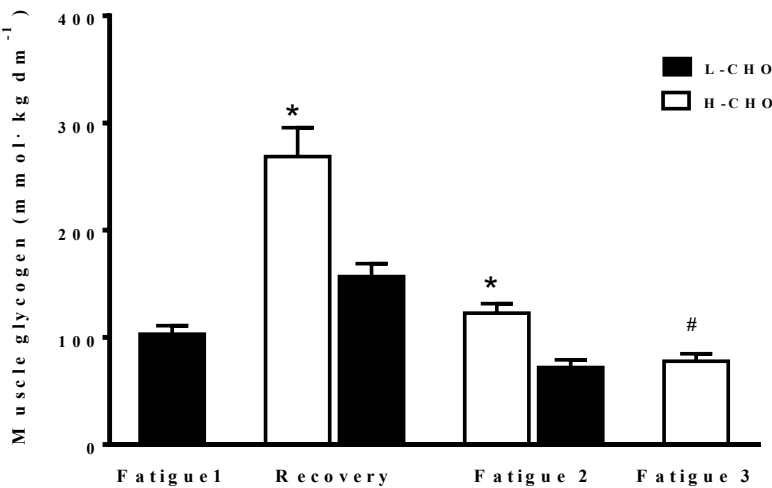


Figure 1. A schematic representation of the study protocol †, body mass assessment; *, fluid provision; #, expired gas and blood sample; ▽, muscle biopsy during L-CHO; ▼, muscle biopsy during H-CHO; F1, fatigue in Run-1; F2, fatigue in L-CHO; F3, fatigue in H-CHO; dashed columns, warm-up; black column, run time to exhaustion in L-CHO trial; grey column, extended run time to exhaustion with H-CHO treatment during Run-2.

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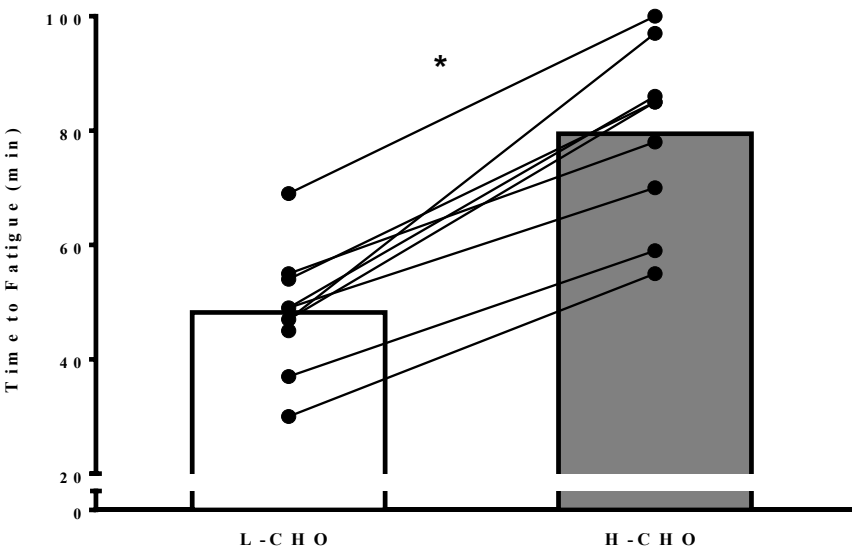
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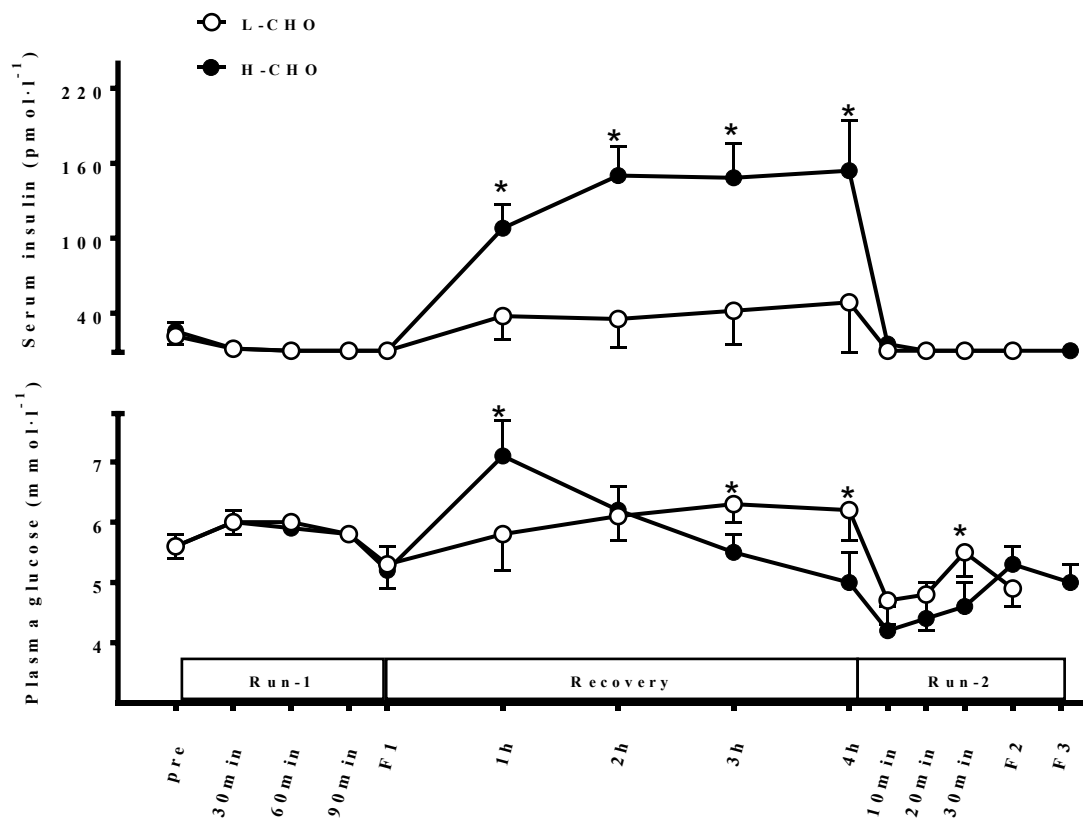


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707 **Figure 2-A.** Muscle glycogen concentrations at the end of Run-1 (F1), at the end of 4
708 h recovery, time to exhaustion with L-CHO treatment (F2) and time to exhaustion
709 with H-CHO treatment (F3). **Figure 2-B,** mean and individual run times to exhaustion
710 following the ingestion of L-CHO or H-CHO during 4 h recovery. Values are means
711 \pm CI. *, values different between L-CHO and H-CHO ($p < 0.01$). #, values different
712 between F2 and F3 within the H-CHO treatment ($p < 0.01$).

713



714
715

716 **Figure 3.** Plasma glucose and serum insulin concentrations during Run-1, recovery
717 and Run-2 with L-CHO or H-CHO treatments. Values are mean \pm CI. *, values
718 different between L-CHO and H-CHO ($p < 0.05$). F1, time to exhaustion during Run-
719 1; F2, time to exhaustion with L-CHO treatment; F3= time to exhaustion with H-CHO
720 treatment.

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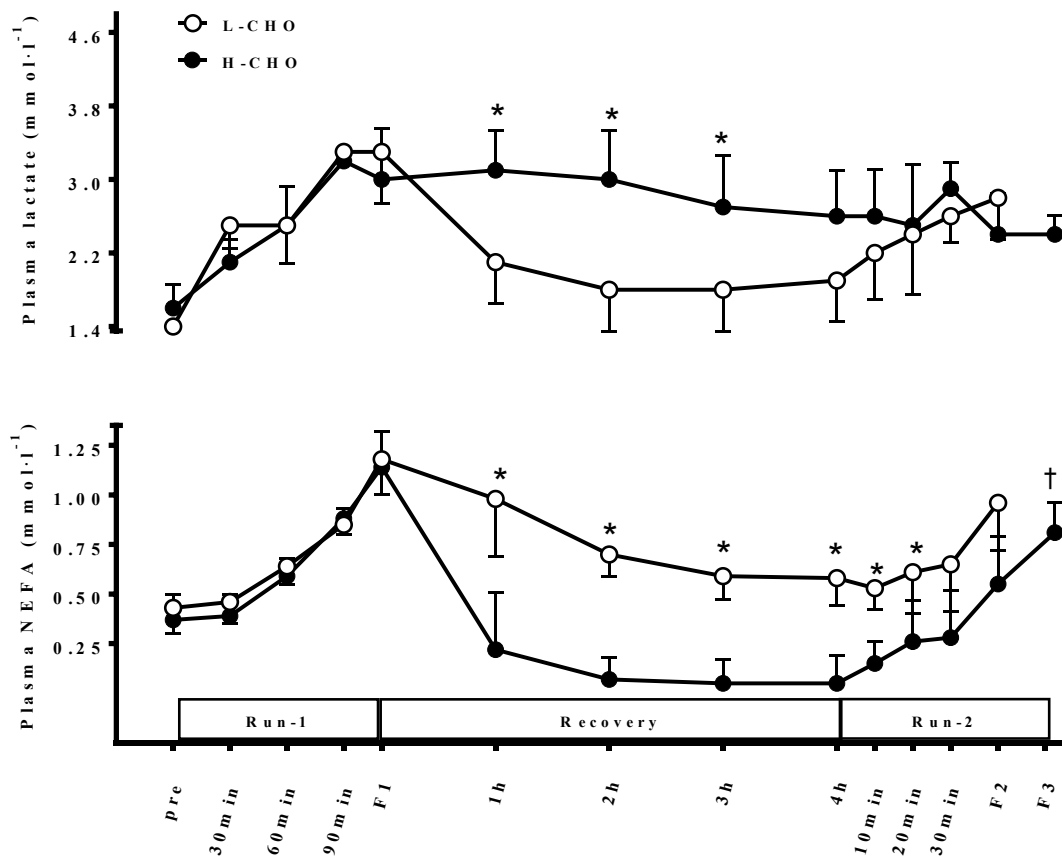


Figure 4. Plasma NEFA and lactate concentrations during Run-1, recovery and Run-2 with L-CHO or H-CHO treatments. Values are mean \pm CI. *, values different between L-CHO and H-CHO ($p < 0.01$). †, values different from F2 to F3 in H-CHO treatment. F1, time to exhaustion during Run-1; F2, time to exhaustion with L-CHO treatment; F3, time to exhaustion with H-CHO treatment.

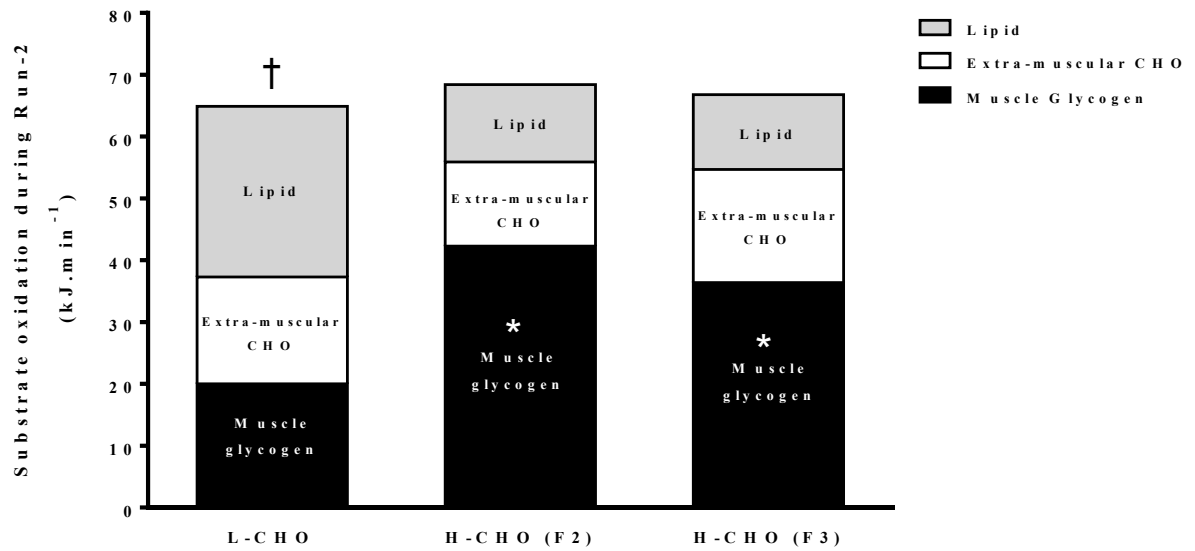


Figure 5. The contribution of muscle glycogen, extra-muscular carbohydrate (CHO) and lipids to total substrate metabolism ($\text{kJ}\cdot\text{min}^{-1}$) during Run-2 with L-CHO or H-CHO treatments. *, Muscle glycogen values different between L-CHO and H-CHO ($p < 0.05$). † = lipid values different between L-CHO and H-CHO treatments. ($p < 0.05$), F1, time to exhaustion during Run1; F2, time to exhaustion with L-CHO treatment; F3, time to exhaustion with H-CHO treatment.